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Abstract: The altitudinal gradient involves changes of the partial pressures of atmospheric gases such as CO2. This omnipresent phenomenon likely represents an evolutionary selective agent. We asked whether high altitude plant species had evolved specific response strategies to cope with high altitude pressure conditions. Plants of the high altitude species Arabis alpina and the low altitude species Arabidopsis thaliana were cultivated in growth chambers with high altitude pressure conditions (corresponding to 3000 m a.s.l.) and low altitude conditions (560 m). In both species, high altitude conditions resulted in the narrowing of stomatal aperture as well as a decrease in leaf area and weight. A. alpina produced significantly more stomata under high altitude conditions compared to low altitude conditions, while A. thaliana did not. Under low altitude conditions, however, stomatal density of A. alpina was smaller compared to A. thaliana. The increase in stomatal density of A. alpina was strongly related to the decrease in the partial pressure of CO2 under high altitude conditions. Thus, the adaptation of the high altitude plant A. alpina to high altitude pressure conditions does not consist in a genetically fixed elevated stomatal density but in a different response strategy of stomatal development to environmental factors compared to the lowland plant A. thaliana. A. alpina developed stomata largely uncoupled from other environmental factors than CO2. The increased stomatal density of A. alpina may ensure an optimal CO2 supply during the periods of favourable weather conditions for photosynthesis that are relatively rare and short in the alpine life zone.

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Arabis alpina and *Arabidopsis thaliana* have different stomatal development strategies in response to high altitude pressure conditions

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Abstract

The altitudinal gradient involves changes of the partial pressures of atmospheric gases such as CO_2. This omnipresent phenomenon likely represents an evolutionary selective agent. We asked whether high altitude plant species had evolved specific response strategies in order to cope with high altitude pressure conditions. Plants of the high altitude species *Arabis alpina* and the low altitude species *Arabidopsis thaliana* were cultivated in growth chambers with high altitude pressure conditions (corresponding to 3000 m a.s.l.) and low altitude conditions (560 m). In both species, high altitude conditions resulted in the narrowing of stomatal aperture as well as a decrease in leaf area and weight. *A. alpina* produced significantly more stomata under high altitude conditions compared to low altitude conditions, while *A. thaliana* did not. Under low altitude conditions, however, stomatal density of *A. alpina* was smaller compared to *A. thaliana*. The increase in stomatal density of *A. alpina* was strongly related to the decrease in the partial pressure of CO_2 under high altitude conditions. Thus, the adaptation of the high altitude plant *A. alpina* to high altitude pressure conditions does not consist in a genetically fixed elevated stomatal density but in a different response strategy of stomatal development to environmental factors compared to the lowland plant *A. thaliana*. *A. alpina* developed stomata largely uncoupled from other environmental factors than CO_2. The increased stomatal density of *A. alpina* may ensure an optimal CO_2 supply during the periods of favourable weather conditions for photosynthesis, that are relatively rare and short in the alpine life zone.

Keywords: CO_2 partial pressure, evolutionary adaptation, stomatal aperture, stomatal density, stomatal index, vapour pressure deficit

Introduction

Mountains are islands of low atmospheric pressure bounded by an ocean of lowlands with higher pressure conditions. This difference in atmospheric pressure exists independently of regional peculiarities or temporal variation (Körner 2003; Nagy and Grabherr 2009) and may therefore act as an evolutionary selective agent. Especially the decrease of the partial pressure of CO_2 with increasing altitude could exert a selective force on plants (Ward and Strain 1997; Ward et al. 2000) and enhance their stomatal development in order to optimise carbon gain (Ward and Kelly 2004; Gerhart and Ward 2010). In fact, the study of the morphology of plant species of high and low altitudes revealed that high altitude species had higher adaxial stomatal density (SD) compared to congeneric species of low altitudes (Körner et al. 1989). Furthermore, for different species it has been found that plants growing at high altitudes have increased (adaxial) SD compared to individuals of the same species but growing at lower altitudes (e.g. Woodward 1986; Körner et al. 1989; Kouwenberg et al. 2007) suggesting that plants increase their SD to compensate for the reduced partial pressure of CO_2 (Kouwenberg et al. 2007). Interestingly, this difference in SD remained when conspecific plants originating from different altitudes were grown under control conditions (e.g. Woodward 1986; Hovenden and Brodribb 2000) indicating that this response could be at least partially genetically controlled (Hovenden and Schimanski 2000; Zhang et al. 2012). However, the positive relationship between altitude and SD was not observed in all the cases studied. In the tropical mountains of New Guinea, Körner et al. (1983) found that the stomatal index (SI) of woody species, i.e. the ratio of stomata to non-stomatal epidermal cells, was smaller at high altitudes than the SI of (other) species at lower altitudes. Other studies revealed that SD was independent of altitude or even decreased with increasing altitude (Körner et al. 1986; Hultine and Marshall 2000; Greenwood et al. 2003; Hu et al. 2015). Furthermore, Qiang et al. (2003) found an increase in SD up to a certain altitude followed by a decrease at higher altitudes. These authors argued that SD and SI may relate to specific local features of the environment, especially to irradiance (and temperature), rather than to the world-wide altitudinal gradient of atmospheric pressure (Körner et al. 1983; Körner et al. 1989; Greenwood et al. 2003; Qiang et al. 2003).

In a meta-study, Royer (2001) found that SD and SI were negatively correlated to the concentration of CO_2 in about half the species responses. This relationship was particularly consistent when fossil or herbarium leaves were compared to modern leaves. Royer (2001) argued for a genetic adaptation of plants to CO_2 concentrations in terms of stomatal development. Accordingly, SD and SI have been used as a proxy to reconstruct paleoatmospheric CO_2 levels (e.g. Woodward 1987; Royer et al. 2001; Stults et al. 2011). However,

52 the study of Royer (2001) also revealed that the negative relationship between CO_2 concentration and SD or SI is
53 not a universal pattern and is highly species specific.
54 The presumably genetically controlled relationship between CO_2 concentration and SD or SI suggests,
55 despite the aforementioned objections, that plants will respond with an increase in SD and SI to the decrease in
56 the partial pressure of CO_2 with altitude. However, in distinction from a change of CO_2 concentration under
57 stable pressure conditions, the decrease of partial pressure of CO_2 with altitude involves at the same time the
58 decline of the partial pressure of the other atmospheric gases such as oxygen and water vapour. It further entails
59 the increase of the diffusion rates of gases due to decreasing atmospheric pressure, which may compensate for
60 the effect of lower partial pressure of CO_2 (Gale 1972) and enhance leaf transpiration (Smith and Geller 1979;
61 Körner 2003). In contrast, low air temperatures at higher altitudes reduce the diffusion rate (Kouwenberg et al.
62 2007) and counteract the diffusion increase due to reduced atmospheric pressure. Consequently, these interacting
63 mechanisms may obscure the direct response of plants to the decreased partial pressure of CO_2 in the field and
64 this response may be better detected with experiments simulating high altitude pressure conditions (Woodward
65 1986). However, while numerous experiments were conducted in order to study the response of plants to
66 variations in CO_2 concentrations, we only know of the study of Woodward and Bazzaz (1988) that investigated
67 the stomatal response of plants to altered atmospheric pressure under experimental conditions. They detected an
68 increase in SD as well as in SI when the partial pressure of CO_2 declined from 34 Pa to 22.5 Pa and the CO_2
69 mole fraction remained fixed (Woodward and Bazzaz 1988). These results strongly argue in favour of a central
70 role of CO_2 partial pressure decreases with altitude on SD and SI (Kouwenberg et al. 2007). However, in the
71 study of Woodward and Bazzaz (1988) plants originating from an altitude below 900 m were cultivated at a
72 partial pressure of CO_2 (22.5 Pa) corresponding to an altitude of *c.* 3300 m. For this study, we cultivated a high
73 altitude species, *Arabis alpina* L. originating from 3000 m a.s.l., under high (3000 m) and low altitude (560 m)
74 pressure conditions and compared its response in terms of SD and SI, as well as stomatal aperture, leaf area and
75 biomass to the closely related low altitude species *Arabidopsis thaliana* (L.) Heynh. We hypothesised that *A.*
76 *alpina* will show generally higher SD than *A. thaliana* and that SD and SI of both species will be increased under
77 high altitude pressure conditions compared to low altitude conditions. Due to the lower availability of CO_2 as the
78 major photosynthetic substrate, we expected that in both species the biomass production under high altitude
79 pressure conditions will be reduced compared to low altitude conditions but that the reduction will be relatively
80 smaller in *A. alpina* than in *A. thaliana*.
81

Materials and methods

Plant material

Arabis alpina is a perennial species with an arctic-alpine distribution occurring in the arctic regions as well as the mountain regions of Europe, northern Africa and western Asia (Hess et al. 1970). In the European Alps, *A. alpina* colonises open habitats, on rather humid, stony and ordinarily calcareous soils such as scree slopes, boulders, or rock crevices (Hess et al. 1970). *Arabis alpina* has a wide altitudinal distribution and can be found in Switzerland from 300 m a.s.l. up to 3250 m; the mean altitude of occurrence is 1708 ± 839 m (n = 1077, data available from Info Flora: www.infoflora.ch). The seeds used for the experiments were collected from a population of *A. alpina* on the Schilthorn summit (2970 m a.s.l.), Bernese Oberland, Switzerland, i.e. at the upper limit of its distribution.

Arabidopsis thaliana is an annual or biennial species originating from the Mediterranean Basin, but nowadays it spreads more or less all over the world (Hess et al. 1970). In Europe, *A. thaliana* grows on loose soils mostly rich in nutrients and poor in carbonates, such as arable fields, road verges, banks, or walls (Hess et al. 1970). In Switzerland, its altitudinal distribution extends from 200 m a.s.l. to 2200 m with a mean altitude of occurrence of 625 ± 364 m (n = 882, data available from Info Flora: www.infoflora.ch). The seeds originate from a laboratory-used *A. thaliana* Columbia (Col-0) wildtype lineage.

Growth conditions and treatments

Plants were cultivated in two custom-made growth chambers (Astromec, Muri b. Bern, Switzerland). One chamber was used for the low altitude cultivation with ambient pressure while air pressure was reduced in the second chamber. The chambers had a volume of 0.21 m^3 (length 700 mm, width 500 mm, height 600 mm), a transparent topside for the illumination, a transparent front door for monitoring and two fittings for tubes on the opposite sidewalls. A vacuum pump (Seco Tiny SV 1003 A; Busch, Maulburg, Germany) working on the rotating vane principle was connected to one tube while a needle valve (B-1RF4; Arbor-Swagelok, Niederrohrdorf, Switzerland) was fixed on the other tube. The vacuum pump continuously worked on a suction capacity of 3 m^3 h^{-1} and the air pressure in the high altitude chamber was reduced by regulating the control valve on the inflow tube. For the low altitude cultivation, the inflow tube was totally open resulting in ambient pressure conditions in this chamber. Air pressure (GPB 2300; Greisinger, Regenstauf, Germany), temperature and relative air humidity (Humicap HM70; Vaisala, Helsinki, Finland) as well as CO_2 concentration (Carbocap GM70; Vaisala, Helsinki, Finland) were continuously measured inside the chambers. The reduction of air

pressure involved a systematic reduction of the partial pressures of CO_2 and H_2O and, since temperature in both chambers was equal, also a systematic relative increase of the vapour pressure deficit (VPD). Since climatic conditions in the room, in which the growth chambers were situated, were not regulated, in both chambers air pressure as well as air temperature and air humidity followed the changes of ambient conditions. This natural variation of climatic conditions among experiments resulted in different levels of temperature, humidity, and therefore VPD, for every experiment preserving at the same time the systematic differences between the two chambers in terms of air pressure and the partial pressures of CO_2 and H_2O. Light was provided with four luminescent tubes type Philips Master TLD 36W/865 (Philips, Amsterdam, The Netherlands) and four luminescent tubes type Osram L 36W/77 Fluora (Osram, Munich, Germany) that were mounted above the chambers. Since the chambers were situated close to each other, they were simultaneously illuminated by the same light sources and, therefore, experienced the same light conditions. Due to the high absorption of the glass pane of the growth chambers, the photosynthetic photon fluence rate (PPFR) on the plant level during the light periods of 12 h was 85 μmol m^{-2} s^{-1} over the waveband 400 to 700 nm (LI-190SA Quantum Sensor; LI-COR, Lincoln NE, U.S.A.). Thus, irradiation was lower than the recommended optimum of approx. 150 μmol m^{-2} s^{-1} for *A. thaliana* cultivation (e.g. Scholl et al. 1998). However, we did not observe any evidence of a light deficit in both species and, in preliminary experiments, *A. thaliana* also flowered after 60 days of cultivation under these light conditions.

Plants were hydroponically cultivated in separate meshed plastic pots (35 mm lower and 50 mm upper diameter, 50 mm height) filled with pellets of expanded clay (diameter 2 to 6 mm). In the growth chambers, the pots were arranged in a grid of 5 by 8, where *A. alpina* alternated with *A. thaliana*. Since the surface of the clay pellets rapidly desiccated in the growth chambers, the seeds were germinated outside of the chambers under ambient pressure conditions for 8 days. After germination, the seedlings consisting of the two cotyledons were transferred to the growth chambers where they were cultivated for 42 days. To assure seedling survival, the plantlets were covered by a transparent plastic hood during the first 14 days of cultivation. After 14 and 28 days, the nutrition solution was substituted to prevent nutrient shortage. The culture medium was composed of 1.5 mM $Ca(NO_3)_2$ $4H_2O$, 1 mM KNO_3, 0.75 mM KH_2PO_4, 0.75 mM $MgSO_4$ $7H_2O$, 30 μM Fe^{3+} ethylenediaminetetraacetic acid, 2 μM H_3BO_3, 0.4 μM $MnCl_2$ $4H_2O$, 0.08 μM MoO_3, 0.07 μM $ZnSO_4$ $7H_2O$ and 0.05 μM $CuSO_4$ $5H_2O$.

Determination of stomatal and whole plant traits

142 The number of experiments conducted as well as the numbers of plants, leaves, leaf surface sections and stomata
143 analysed are given in Online Resource 1. For the determination of leaf area, stomatal density, stomatal index and
144 stomatal aperture, generally the lowermost intact leaves of a rosette were used. However, the two oldest leaves
145 were avoided in order to guarantee that the leaves fully developed under treatment conditions in the growth
146 chambers.

147 For the determination of stomatal aperture, two leaves per plant were cut and rapidly immersed in liquid
148 nitrogen after opening of the growth chambers at the end of an experiment. Afterwards, the leaves were stored at
149 -20 °C until stomatal aperture was measured with the use of an Olympus BX51 Microscope (Olympus, Tokyo,
150 Japan), a SIS View Fire Wire digital camera and Analysis 3.2 image processing software (both Soft Imaging
151 System, Münster, Germany). Ten stomatal apertures per leaf were measured to the nearest 0.01 μm.

152 Since there were hardly any stomata on the adaxial leaf surfaces, probably due to the relatively low light
153 intensity, only abaxial stomatal density (SD) was determined. For this purpose, two or five leaves per plant were
154 cut and impressions of abaxial leaf surfaces were taken using dental impression gel, i.e. a mixture of Xantopren
155 VL Plus and Optosil P Plus (Heraeus Kulzer, Hanau, Germany). From the silicone imprints, transparent, positive
156 imprints were made with customary nail varnish. Stomata were counted for two or four sections of 0.094 mm^2
157 per leaf using the same optical system as described above. For stomatal index (SI), stomata and epidermal cells
158 were counted within sections of 0.04 mm^2.

159 For the determination of leaf area, two leaves per plant were cut, scanned with a resolution of 300 dpi and
160 analysed with Easy Leaf Area (Easlon and Bloom 2014). Preliminary investigations showed that shoot fresh
161 weight and dry weight were highly correlated (r = 0.975, n = 130, Online Resource 2). Thus, only fresh weight
162 was determined for the following experiments. The leaves of each plant were counted and weighed to the nearest
163 0.1 mg instantly after the end of an experiment. For shoot/root ratio, leaf rosettes were cut from the roots and the
164 latter were carefully separated from the clay pellets. Roots and leaf rosettes were oven dried (80 °C, 48 h) and
165 weighed to the nearest 0.1 mg.
166
167 Statistical analyses
168 We tested for treatment effects on all plant traits (stomatal aperture, SD, SI, number of leaves, leaf area, fresh
169 weight) and their differences between species using generalised linear mixed models with ‘species’, ‘treatment’
170 and their interaction term as fixed factors. Depending on the sampling design we included ‘experiment’ (for all
171 response variables except SI), ‘individual’ nested within ‘experiment’ (for SD and leaf area) and ‘leaf’ nested

within 'experiment' (for stomatal aperture) or 'leaf' nested within 'individual' nested within 'experiment' (for SD) as random factors. For SD we used a Poisson error structure and a log link-function and included the log of the area where stomata were counted (0.094 mm^2) as an offset term. All other variables met normality assumptions and a Gaussian error structure was used. To test for treatment effects within species we used orthogonal contrasts.

In order to disentangle the actual driver of changes in plant performance in response to the air pressure treatment, we related partial pressure of CO_2 and vapour pressure deficit to plant traits and their differences between species. For each response variable we used the same model structure as described above, except the fixed factors. As fixed factors we used 'Species', 'CO_2', 'VPD' and the interactions 'Species × CO_2' and 'Species × VPD' and included the mean temperature as covariate.

In all analyses statistical significance of fixed factors was determined using type-II analysis of variance whereas significance of contrasts was determined with t-tests. Statistical analyses and figures were done with R software version 2.15.2 (R Development Core Team 2012).

Results

Experimental conditions

The reduced atmospheric pressure treatment was associated with lower partial pressure of CO_2 (-22 %) and relative humidity (-9 %) but higher vapour pressure deficit (+10 %) compared to low altitude conditions under ambient pressure (Table 1). The climatic conditions of the low pressure treatment (i.e. the high altitude conditions) closely corresponded to the climatic conditions of an alpine habitat of *A. alpina* in the central Swiss Alps (Gemmi Pass, 46.43° N, 7.63° E) as they occur during periods of high irradiance (above 500 W m^{-2}) between mid June and end of August (Vonlanthen et al. 2004 and unpublished data).

Stomata

For both species, the aperture of stomata was *c.* 4 μm under low altitude conditions and significantly smaller (*c.* 3 μm, -24 %) under high altitude conditions (Fig. 1a, Table 2). Narrower stomatal aperture was significantly related to increased VPD (Fig. 3a, Table 4) and to higher partial pressure of CO_2 (Fig. 2a, Table 4).

Under low altitude conditions, stomatal density (SD) was between 110 and 125 stomata per 1 mm^2 leaf surface for both species and significantly increased under high altitude conditions by 18 % for *A. alpina* while *A. thaliana* showed no response (Fig. 1b, Table 2). The different response of SD of the two species to high altitude

conditions was related to their distinct response to the partial pressure of CO_2 (Fig. 2b, Table 4) and VPD (Fig. 3b, Table 4). Separate analyses of the relationships of SD with CO_2 and VPD for each species showed significantly increasing SD with decreasing partial pressure of CO_2 for *A. alpina* only and no significant relationships between SD and VPD for both species (Table 5).

The number of epidermal cells per leaf surface area was around 1050 cells per mm^2 for both species and irrespective of the pressure conditions (Fig. 1c, Table 2). Consequently, the stomatal index (SI), i.e. the number of stomata in a given area divided by the total number of guard cells and other epidermal cells in the same area, was significantly increased under high altitude conditions compared to low altitude conditions in *A. alpina* while it remained unchanged in *A. thaliana* (Fig. 1d, Table 2).

Leaves

After 42 days *A. alpina* developed about 16 leaves whereas *A. thaliana* had approximately 28 leaves. Both species showed no changes in the number of leaves in response to the different pressure conditions (Fig. 1e, Table 3). However, both species developed approximately 15 % smaller leaves under high altitude conditions compared to low altitude conditions (Fig. 1f, Table 3). The reduction in leaf area was most strongly related to VPD (Table 4). However, the significant interaction terms between species and either partial pressure of CO_2 or VPD also indicate that for the two species CO_2 and VPD are not equally related to leaf area (Table 4). Separate analyses showed that for both species leaf area was more strongly related to VPD and significantly less to CO_2 (Figs. 2c, 3c and Table 6), but only in *A. thaliana* the response of leaf area to VPD was significant.

Biomass

Under high altitude conditions, fresh weight of the leaf rosettes was significantly smaller (14 % in *A. thaliana* and 19 % in *A. alpina*) than under low altitude conditions (Fig. 1g, Table 3). This difference was significantly related to VPD (Fig. 3d, Table 4), but not to the partial pressure of CO_2 (Fig. 2d, Table 4). The decline in fresh weight with increasing VPD was more pronounced for *A. alpina* than for *A. thaliana* (Fig. 3d). In similar fashion to the fresh weight of shoots, the fresh weight (not shown) and dry weight of roots was reduced under high altitude conditions (Fig. 1h, Table 3). The shoot/root-ratio was significantly higher for *A. alpina* than for *A. thaliana*, but was not significantly different between treatments (Fig. 1i, Table 3).

Discussion

The reduced atmospheric pressure resulted in a decrease of CO_2 partial pressure and an increase of vapour pressure deficit (VPD) and caused the narrowing of stomatal aperture and a decrease in leaf area as well as shoot and root weight in both species under study. However, *A. alpina* showed increased stomatal density (SD) and a higher stomatal index (SI) under high altitude pressure conditions while in *A. thaliana*, SD and SI were not significantly different between high and low altitude conditions. The increase in SD of *A. alpina* was strongly related to the decrease in the partial pressure of CO_2 but not to the increase in VPD.

Stomata

For both species the aperture of stomata was equally reduced under high altitude conditions compared to low altitude conditions. The narrowing of the stomatal aperture was highly related to the increased VPD under high altitude conditions as plants close their stomata in response to a reduction in the concentration of water vapour in the atmosphere (Buckley 2005; Belin et al. 2010) or to an increase in the transpiration rate (Mott and Parkhurst 1991), respectively. At the same time, the results also suggest that plants tend to close the stomata with increasing partial pressure of CO_2 which is in line with the findings of studies showing that stomatal aperture and/or conductance increases with a decreasing concentration of CO_2 (e.g. Mott 1990; Hashimoto et al. 2006; Hu et al. 2010). However, the reduced stomatal aperture under high altitude conditions indicates that the increased water vapour gradient between leaf and atmosphere was a stronger signal for stomatal closure than the opposing effect of a reduced partial pressure of CO_2. These findings appear contradictory to those of Merilo et al. (2014) who found that the simultaneous increase of VPD and decrease of the concentration of CO_2 resulted in stomatal opening of *A. thaliana*. However, in their study the concentration of CO_2 was rapidly reduced from 400 to 50 ppm. Such a great and rapid decrease of the concentration of CO_2 does not occur in nature and, therefore, the stomatal response to this signal may be unrealistic. Our results rather correspond to the findings of Talbott et al. (2003) showing that relative air humidity is a key environmental factor mediating the changes in stomatal sensitivity to CO_2. In short, our results show that the plants responded to a relatively small increase in VPD (10 %) by a substantial narrowing of stomatal aperture (-24 %) even though the water supply of the roots was totally unrestricted.

Contrary to our expectations, SD of *A. alpina* was higher compared to *A. thaliana* only under high altitude conditions and *A. thaliana* did not develop more stomata under high altitude compared to low altitude conditions. The abaxial SD of *A. alpina* was significantly increased under high altitude conditions, which actually correspond to its home pressure conditions, compared to low altitude conditions. This finding is in line

with the study of Körner et al. (1989) who found that the high altitude species *A. alpina*, *Linaria alpina* (L.) Miller and *Oxyria digyna* (L.) Hill produce fewer stomata per unit abaxial leaf area when transplanted to lower altitudes. This inverse relationship between SD and partial pressure of CO_2 further corresponds with numerous studies that showed that plants decrease their SD in response to an increase in partial pressure or in concentration of CO_2 (e.g. Woodward and Bazzaz 1988; Woodward and Kelly 1995; Royer 2001). Since there was no significant difference in the epidermal cell density of *A. alpina* between treatments, the increased SD under high altitude conditions was not the result of smaller epidermal cells, but due to a higher proportion of meristemoid epidermal cells developing into guard cells.

Contrary to *A. alpina*, the SD of *A. thaliana* was not significantly different between low and high altitude conditions. Since epidermal cell density was not significantly different either, essentially the same proportion of epidermal cells was converted into guard cells resulting in a similar SI under low and high altitude conditions for this species. Thus, our results would suggest that the development of stomata of the lowland species *A. thaliana* was insensitive to changes in the partial pressure of CO_2. This would be in contrast to Woodward et al. (2002) and Teng et al. (2006) who showed that *A. thaliana* responded to a doubling of the CO_2 concentration with a significant decrease in SD. However, in these studies, relative air humidity was held constant. Woodward et al. (2002) further argued that soil moisture may modify the stomatal response to changes in CO_2 concentration. They found that in *A. thaliana* Col-0 with a doubling of the CO_2 concentration the decrease in SD was significantly higher under dry soil conditions compared to humid soils. This behaviour suggests that *A. thaliana* decreases SD in order to minimise water loss under water stress conditions. Transferred to our experiments, we argue that under high altitude pressure conditions, *A. thaliana* primarily responded to the increased VPD and to the higher diffusion rate of water vapour by keeping SD constant despite of the reduced partial pressure of CO_2. In fact, studies with mutations and transgenic plants of *A. thaliana* showed that the reduction of SD enhances drought resistance in *A. thaliana* (Yoo et al. 2010, Wang et al. 2012; Xie et al. 2012).

Light intensity is known to regulate the formation of stomata and increased light quantity can positively stimulate changes in stomatal numbers (Casson and Hetherington 2010). Therefore, the missing response of *A. thaliana* to high altitude conditions in terms of stomatal density could be an artefact of the relatively low light intensity used in our experiments (if the low light intensity suppressed the stomatal response of this species to changes in VPD and/or CO_2). Casson et al. 2009 cultivated *A. thaliana* at even lower light intensities (50 µmol m^{-2} s^{-1}), similar temperature (22°C), but higher relative air humidity (70 %) and therefore lower VPD compared to our experiments. They determined abaxial densities of around 100 stomata mm^{-2}, thus slightly less than in our

292 experiments (approx. 120 stomata mm^{-2}). These results indicate, that *A. thaliana* is basically able to produce
293 more stomata under low light intensities. Therefore, we have no reason to assume that the lacking response of
294 stomatal density of *A. thaliana* under high alpine conditions is merely an artefact of the relatively low light
295 intensity. We rather believe that it is the result of increased VPD as suggested by the statistical analyses.
296 Taken together, our results strongly indicate that the high altitude species *A. alpina* responded to the high
297 altitude CO_2 conditions irrespective of the increased VPD while the response of the lowland species *A. thaliana*
298 seems to primarily depend on air humidity conditions (increased VPD and diffusion rate of water vapour).
299
300 Leaves and biomass
301 The reduced weight of the plants cultivated under high altitude pressure conditions is in line with numerous
302 studies showing that the biomass production of plants was decreased when grown at subambient concentrations
303 of CO_2 (e.g. Ward and Strain 1997; Cowling and Sage 1998; Hovenden and Schimanski 2000). However, our
304 results show that the reduction of biomass was due to increased VPD under high altitude conditions rather than
305 to the decreased partial pressure of CO_2. Since the number of leaves did not differ between treatments, the
306 weight of the leaf rosettes (shoot weight) was primarily a function of leaf area and/or leaf thickness. The
307 reduction of leaf area under high altitude conditions was more strongly related to VPD than to the partial
308 pressure of CO_2. Thus, our results suggest that the reduction of shoot weight under high altitude conditions was
309 due to the reduction in leaf area that resulted from the increased VPD.
310 Epidermal cell density was not significantly different between treatments, indicating that the decreased
311 leaf area cannot be due to smaller epidermal cells, i.e. decreased cell expansion, but must primarily be the result
312 of a reduced total cell number under high altitude conditions. Even though it is known that moderate soil water
313 deficit (Wuyts et al. 2012) or mild osmotic stress (Skirycz et al. 2011) may cause a reduction in leaf surface area
314 due to reduced cell numbers in the epidermis, to the best of our knowledge, there are no studies showing a plant
315 response in cell proliferation to relative air humidity or VPD, respectively. Nevertheless, our analyses suggest
316 that increased VPD alone, i.e. without soil water stress, reduced cell proliferation and leaf area. However,
317 Ranasinghe and Taylor (1995) as well as Masle (2000) showed that elevated concentrations of CO_2 increased the
318 cell division rates compared to ambient CO_2 concentration in *Phaseolus vulgaris* L. and *Triticum aestivum* L.,
319 respectively. Thus, it appears to be plausible that the reduced partial pressure of CO_2 under high altitude
320 conditions may also have contributed to the diminished cell proliferation. In sum, we conclude that the smaller
321 leaf area of the plants grown under high altitude conditions were the result of a decreased cell proliferation

during the first phase of lamina formation in response to increased VPD and/or low partial pressure of CO_2. This suggests that increased VPD alone, or combined with low partial pressure of CO_2, may contribute to the dwarfism of plants at high altitudes by means of its limiting effect on cell proliferation.

Based on the increased SD in *A. alpina* under high altitude conditions, one would expect that *A. alpina* should show higher biomass production compared to *A. thaliana*. However, this was not the case. The reductions in leaf area as well as in shoot and root weight under high altitude conditions were similar in *A. alpina* and *A. thaliana*. We suppose that the lack of a differential biomass response of the two species to high altitude pressure conditions is due to the low light intensity of *c.* 85 µmol m^{-2} s^{-1} in our experiments. It is known that at this range of light intensity the photosynthetic CO_2 assimilation is limited by the low rate of RuBP regeneration due to the reduced production of NADPH and ATP in the light reaction (Farquhar et al. 1980; Terashima et al. 1995). Under *in situ* conditions where high light intensities occur, the increased SD of *A. alpina* most likely represents an advantage for photosynthesis and plant growth (Tanaka et al. 2013). Körner (2003) stated that the major limitation of alpine plant photosynthesis is the photosynthetically active quantum flux density. During periods of high quantum flux density, i.e. conditions of high solar radiation, VPD is inevitably high. Furthermore, increased leaf thickness due to a greater amount of photosynthesising tissue is one of the most universal trends in high altitude plant species (Körner 2003). In addition, it has been shown that the lateral diffusion of CO_2 in leaves may represent a limiting factor for photosynthesis when stomata are widely spaced (Morison et al. 2005; Büssis et al. 2006). Thus, *A. alpina* may profit from periods of high quantum flux density and temperature due to its increased SD that ensures an optimum CO_2 supply of the photosynthetically active tissues even when the stomata are partly closed to reduce water loss. The CO_2 uptake of the low altitude species *A. thaliana*, however, would be limited under the same weather conditions due to relatively low SD and stomatal aperture. Accordingly, the increase of SD of *A. alpina* under high altitude conditions appears to be an efficient response to the optimum weather conditions for photosynthesis during clear summer days that are relatively rare in the alpine life zone.

Conclusions

To sum up, the data show that the adaptation of the high altitude plant *A. alpina* to high altitude pressure conditions does not consist in a genetically fixed elevated density of stomata (SD) but in a different response strategy of stomatal development to environmental factors compared to the lowland plant *A. thaliana*. In *A. alpina*, the stomatal response to low air pressure tightly followed the reduced partial pressure of CO_2 but was not

related to increased vapour pressure deficit (VPD), while in *A. thaliana*, the apparently counterdirectional effects of decreased partial pressure of CO_2 and increased VPD neutralised each other and SD for this species remained stable with changing air pressure conditions. We conclude, that the increased SD of *A. alpina* may ensure an optimal CO_2 supply of the photosynthetically active tissue during bright and warm periods when VPD is elevated due to high irradiance and temperatures and when, consequently, stomatal aperture may be narrowed. This may be especially important at the alpine life zone where favourable weather conditions for photosynthesis are relatively rare and short. The increased SD may lead to higher growth rates and increased fitness compared to plants that are not able to increase SD under high altitude conditions.

In conclusion, our study suggests that there exists a trade-off between carbon gain and water loss not only at the level of the control of stomatal aperture but also at the level of the control of stomatal development. At high altitudes, the continuing deprivation of CO_2 as the major photosynthetic substrate seems to be a strong evolutionary agent able to influence the trade-off between stomatal development strategies in the long-term. In the high altitude plant *A. alpina*, the trade-off appears to be resolved in favour of long-term optimisation of carbon uptake by increased stomatal development. In this species, the control of stomatal development seems to be uncoupled from air humidity and the plants respond to increased VPD solely by short-term regulation of stomatal aperture. In the lowland plant *A. thaliana*, however, the result of this trade-off depends on the respective predominating conditions of CO_2 and air humidity. These different stomatal development response strategies may explain the inconsistency in the response of stomatal density and index to changes in partial pressure or concentration of CO_2 as observed in previous studies.

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Additional supporting information in the online version of this article (see "Supplementary Material") **contains the following:**

ESM_1 – Overview of experiments and data collection.

ESM_2 **–** Correlation between dry and fresh weight of shoots.

Table 1 Climatic conditions in the growth chambers of the experiment either under ambient atmospheric pressure (low altitude), under low pressure (high altitude), and in an open alpine grassland in the western part of the central Swiss Alps (2335 m a.s.l., 46.43° N, 7.63° E) during periods of bright weather.

	growth chambers		
	low altitude [1)]	high altitude [1)]	alpine grassland [2)]
Atmospheric pressure	952.0 ± 8.1 hPa	692.8 ± 12.4 hPa	756 hPa [6)]
CO_2 partial pressure	39.3 ± 2.1 Pa	30.6 ± 1.3 Pa	29.1 Pa [7)]
Relative air humidity	56.9 ± 5.8 % [4)]	51.6 ± 4.5 % [5)]	49.6 ± 11.6 % [8)]
Vapour pressure deficit [3)]	10.1 ± 1.1 hPa	11.1 ± 0.9 hPa	13.4 ± 5.3 hPa [8)]
Temperature	20.0 ± 1.8 °C	19.5 ± 1.7 °C	20.9 ± 3.6 °C [8)]
Radiation	ca. 85 μmol s^{-1} m^{-2}		733.7 ± 142.1 W m^{-2} [9)]

[1)] Means and standard deviations of hourly measures during light periods of eight experiments (duration 42 days each), [2)] means and standard deviations of hourly measures during periods of high radiation (above 500 W m^{-2}) between mid June and end of August 2002 (Vonlanthen et al. 2004 and unpublished data). [3)] approximation of saturation vapour pressure applying the Magnus formula (e.g. Alduchov and Eskridge 1996), [4)] during the first 14 days of culture approx. 90 %, [5)] during the first 14 days of culture approx. 85 %, [6)] calculated using the barometric formula, [7)] calculated applying the formula given by Kouwenberg et al. 2007, p. 224, [8)] measured at 0.15 m above ground, [9)] measured at 0.5 m above ground. Mean CO_2 mole fraction was 395.0 ± 22.5 ppm during the eight experiments.

Table 2 Results of mixed model analyses testing stomatal responses of *Arabis alpina* and *Arabidopsis thaliana* to high and low altitude pressure conditions. For stomatal aperture $n = 800$, for stomatal density $n = 1436$, for epidermal cell density and stomatal index $n = 80$

		Stomatal aperture		Stomatal density		Epidermal cell density		Stomatal index	
Factor	d.f.	X^2	*P*-value	X^2	*P*-value	*F*	*P*-value	*F*	*P*-value
Treatment	1	558.6	<0.001	21.9	<0.001	0.61	0.44	8.7	0.004
Species	1	28.9	<0.001	0.5	0.46	0.01	0.92	3.3	0.074
Treatment x Species	1	2.2	0.14	37.4	<0.001	0.38	0.54	13.0	<0.001

532 **Table 3** Results of mixed model analyses testing plant trait responses of *Arabis alpina* and *Arabidopsis thaliana*
533 to high and low altitude pressure conditions. For number of leaves $n = 251$, for leaf area $n = 480$, for shoot
534 weight $n = 329$, for root weight and shoot/root-ratio $n = 78$
535

		Number of leaves		Leaf area		Shoot (fresh weight)		Root (dry weight)		Shoot/root-ratio (dry weight)	
Factor	d.f.	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value	*F*	*P*-value	*F*	*P*-value
Treatment	1	2.3	0.132	1042.1	<0.001	31.0	<0.001	5.13	0.026	0.93	0.34
Species	1	448.2	<0.001	40.9	<0.001	42.0	<0.001	0.72	0.40	50.05	<0.001
Treatment x Species	1	0.8	0.38	8.2	0.004	1.9	0.16	1.08	0.30	0.06	0.80

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539 **Table 4** Results of mixed model analyses testing plant trait responses of *Arabis alpina* and *Arabidopsis thaliana*

540 to changes in partial pressure of CO_2 (CO_2) and vapour pressure deficit (VPD)

541

		Stomatal aperture		Stomatal density		Number of leaves		Leaf area		Shoot (fresh weight)	
Factor	d.f.	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value
Species	1	29.2	<0.001	0.5	0.47	457.0	<0.001	1216.4	<0.001	42.6	<0.001
CO_2	1	4.3	0.039	21.2	<0.001	0.1	0.78	0.7	0.40	1.4	0.24
VPD	1	24.0	<0.001	0.7	0.40	<0.1	0.95	7.5	0.006	6.2	0.013
Species x CO_2	1	0.4	0.51	51.3	<0.001	0.3	0.56	43.8	<0.001	0.2	0.63
Species x VPD	1	3.8	0.051	21.9	<0.001	6.5	0.011	73.7	<0.001	10.5	0.001

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Table 5 Stomatal density responses to CO_2 and VPD for each species.

Results of mixed model analyses testing stomatal density responses of *Arabis alpina* and *Arabidopsis thaliana* to changes in partial pressure of CO_2 (CO_2) and vapour pressure deficit (VPD)

		A. alpina		*A. thaliana*	
Factor	d.f.	X^2	*P*-value	X^2	*P*-value
CO_2	1	30.87	<0.001	0.34	0.56
VPD	1	0.80	0.37	0.18	0.67

555 **Table 6** Leaf area responses to CO_2 and VPD for each species.

556 Results of mixed model analyses testing leaf area responses of *Arabis alpina* and *Arabidopsis thaliana* to

557 changes in partial pressure of CO_2 (CO_2) and vapour pressure deficit (VPD)

558

		A. alpina		*A. thaliana*	
Factor	d.f.	X^2	*P*-value	X^2	*P*-value
CO_2	1	0.10	0.749	0.03	0.858
VPD	1	3.78	0.052	10.68	0.001

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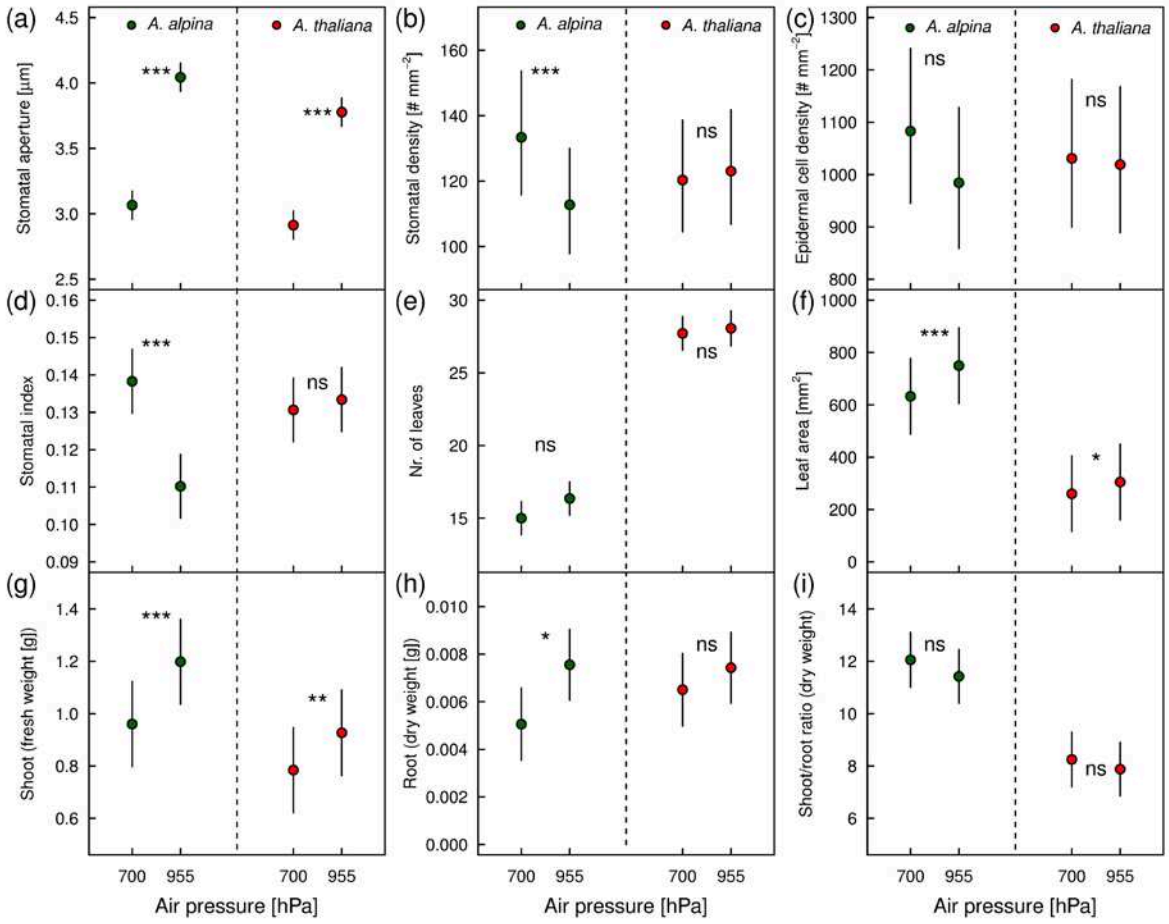
Figure captions

Fig. 1 Stomatal and growth responses of *Arabis alpina* and *Arabidopsis thaliana* grown under high and low altitude pressure conditions. Low (955 hPa) and high (700 hPa) altitude pressure conditions reflect air pressure conditions at *c.* 560 m and 3000 m a.s.l. respectively. Displayed are means and 95 % CIs and results of orthogonal contrasts testing for species-specific responses of stomatal aperture (a), stomatal density (b), epidermal cell density (c), stomatal index (d), number of leaves (e), leaf area (f), fresh weight of shoots (g), dry weight of roots (h) and shoot to root ratio based on dry weight (i) between low and high altitude pressure conditions (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns = not significant).

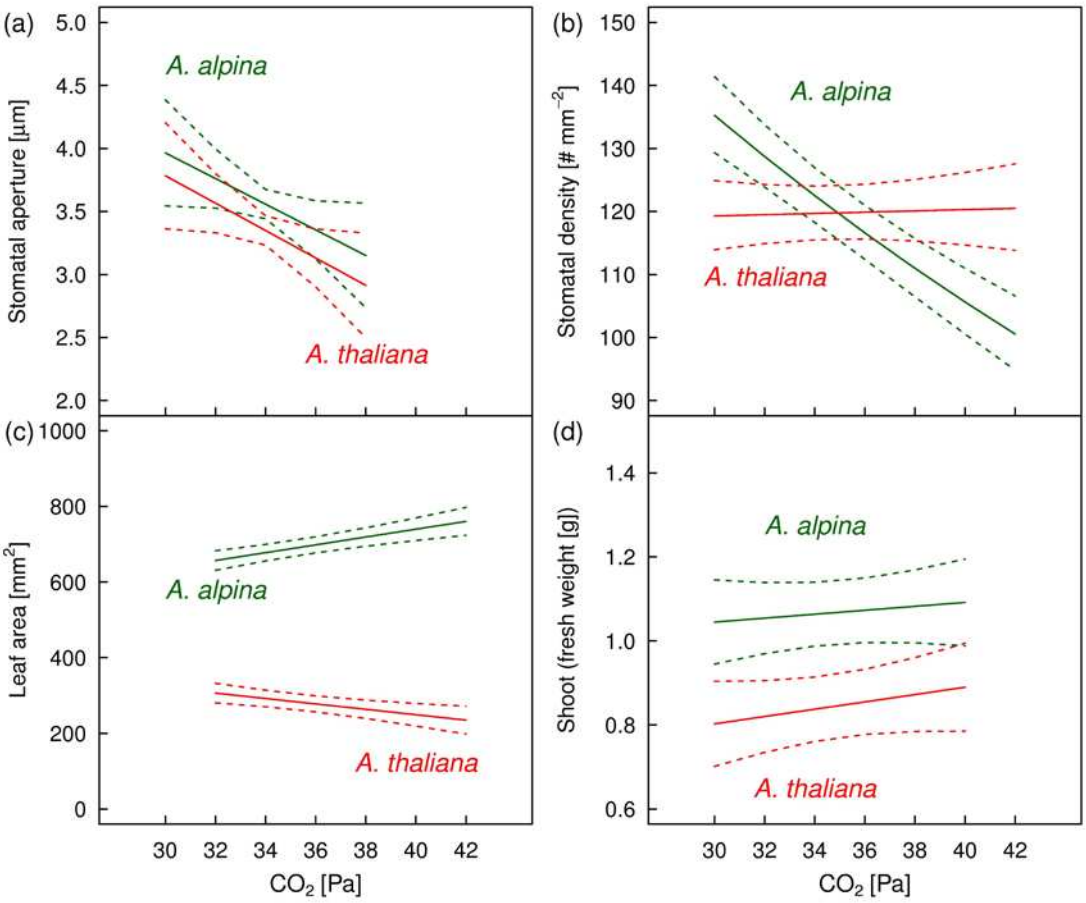
Fig. 2 Plant trait responses of *Arabis alpina* and *Arabidopsis thaliana* to changes in partial pressure of CO_2 as a response to alterations in air pressure. Displayed are predicted means and 95 % CIs of the partial effect of partial pressure of CO_2 on stomatal aperture (a), stomatal density (b), leaf area (c) and fresh weight of shoots (d). The graphs show the relationship of each response variable for the two species with the changes in partial pressure of CO_2 observed due to the experimental manipulation of air pressure conditions. The displayed relationships represent the model output for the interaction 'Species x CO_2' after fitting the full model including the three main effects of species, partial pressure of CO_2 and vapour pressure deficit, and the two interaction terms 'Species x CO_2' and 'Species x VPD'. The corresponding statistical results are shown in Table 4.

Fig. 3 Plant trait responses of *Arabis alpina* and *Arabidopsis thaliana* to changes in vapour pressure deficit as a response to alterations in air pressure. Displayed are predicted means and 95% CIs of the partial effect of vapour pressure deficit (VPD) on stomatal aperture (a), stomatal density (b), leaf area (c) and fresh weight of shoots (d). The graphs show the relationship of each response variable for the two species with the changes in VPD observed due to the experimental manipulation of air pressure conditions. The displayed relationships represent the model output for the interaction 'Species x VPD' after fitting the full model including the three main effects of species, partial pressure of CO_2 and vapour pressure deficit, and the two interaction terms 'Species x CO_2' and 'Species x VPD'. The corresponding statistical results are shown in Table 4.

Fig. 1


(a)
Stomatal aperture [µm]
A. alpina
A. thaliana
***
***
(b)
Stomatal density [# mm^{-2}]
***
ns
(c)
Epidermal cell density [# mm^{-2}]
ns
ns
(d)
Stomatal index
***
ns
(e)
Nr. of leaves
ns
ns
(f)
Leaf area [mm^2]
***
*
(g)
Shoot (fresh weight [g])
***
**
(h)
Root (dry weight [g])
*
ns
(i)
Shoot/root ratio (dry weight)
ns
ns
700
955
Air pressure [hPa]

598 **Fig. 2**



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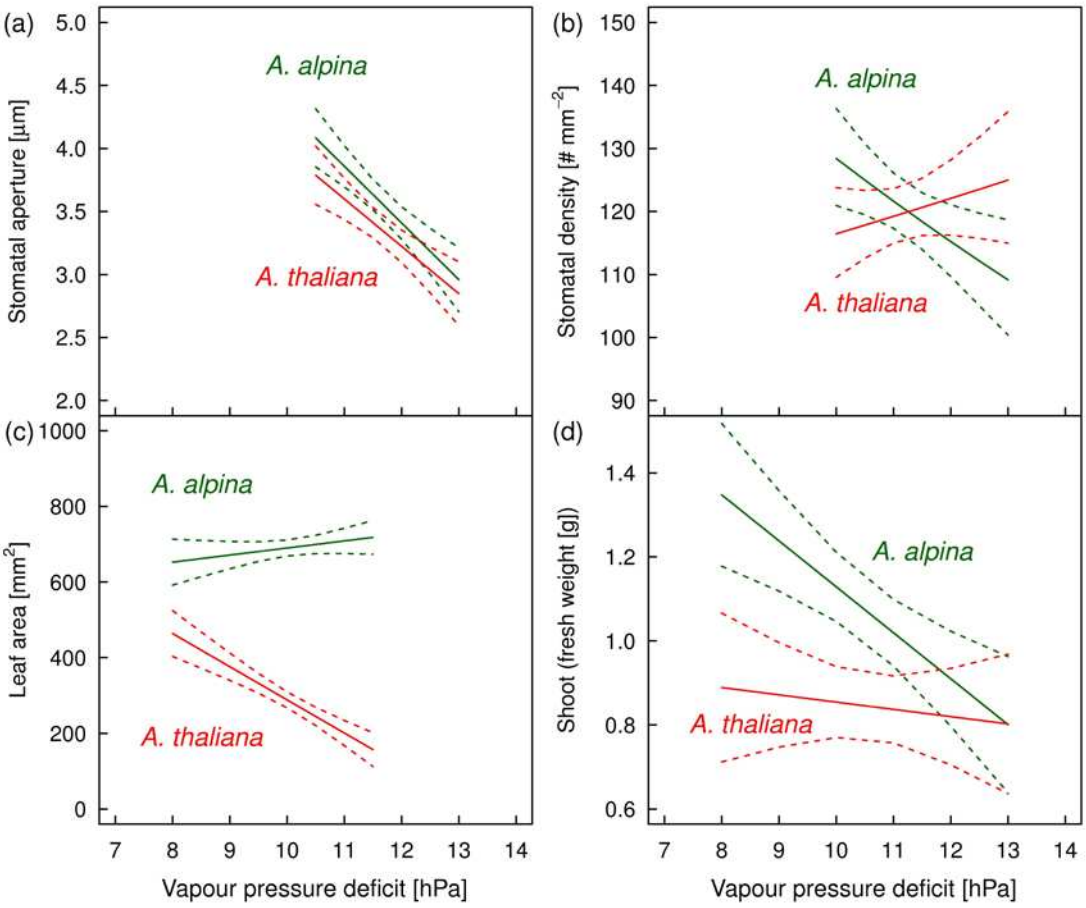
601 **Fig. 3**


(a)
Stomatal aperture [µm]
A. alpina
A. thaliana
(b)
Stomatal density [# mm^{-2}]
A. alpina
A. thaliana
(c)
Leaf area [mm^2]
A. alpina
A. thaliana
(d)
Shoot (fresh weight [g])
A. alpina
A. thaliana
Vapour pressure deficit [hPa]
Vapour pressure deficit [hPa]

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